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Expression of three expansin genes during development and maturation of Kyoho grape berries

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Grape berry;
Véraison;
Softening

Summary

Expansins are cell-wall-localized proteins that induce loosening of isolated plant cell walls *in vitro* in a pH-dependent manner, but exhibit no detectable hydrolase or transglycosylase activity. Three putative expansin cDNAs, *Vlexp1*, *Vlexp2*, and *Vlexp3* were isolated from a cDNA library made from mature berries of the Kyoho grape. Expression profiles of the 3 genes were analyzed throughout berry development. Accumulation of the *Vlexp3* transcript was closely correlated with berry softening, and expression of this gene was detected before véraison and markedly increased at véraison (onset of berry softening). Expression of *Vlexp3* was berry-specific. *Vlexp1* and *Vlexp2* mRNA accumulation began during the expansion stage of berry development and expression increased for both genes during ripening. *Vlexp1* and *Vlexp2* mRNA was detected in leaf, tendril and flower tissues and *Vlexp2* mRNA was additionally detected in root and seed tissues. These findings suggest that the three expansin genes are associated with cell division or expansion and berry ripening. *Vlexp3,* in particular, is most likely to play a role in grape berry softening at véraison.

Introduction

The primary cell wall of plants has been described as a network of cellulose microfibrils embedded in a hemicellulosic polysaccharide matrix that interacts

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to some degree with an additional coextensive matrix of pectin and other less abundant components, including structural proteins (Carpita and Gibeaut, 1993). In dicotyledons, the predominant hemicellulose is xyloglucan, and it is thought that cellulose microfibrils are coated and tethered by a framework of xyloglucan polymers (Hayashi, 1989; McCann et al., 1990). Candidates for mediating hemicellulose modification as a mechanism for cell expansion include cellulase and xyloglucan *endo*-transglycosylase, which have been associated with rapidly expanding tissues (Fry, 1989; Fry et al., 1992; Nishitani and Tominaga, 1992). Neither of these classes of enzymes, however, appeared to cause extension in an *in vitro* assay using isolated cell walls (McQueen-Mason et al., 1992). Instead, a class of proteins called expansins, which causes cell-wall loosening in stress-relaxation assays, and which lacks detectable hydrolytic or transglycosylase activity has recently been identified (McQueen-Mason et al., 1993; McQueen-Mason and Cosgrove, 1994, 1995). It has been proposed that expansins disrupt noncovalent linkages, such as hydrogen bonds, at the cellulose–hemicellulose interface, thereby loosening an important constraint to turgor-driven cell expansion (Cosgrove, 2000).

During fruit ripening, both the pectic and hemicellulosic polymers generally undergo substantial depolymerization and solubilization (Gross and Wallner, 1979; Huber, 1983). Much research has focused on pectin degradation, resulting from the action of the ripening-related enzyme polygalacturonase as the key element underlying the softening process. Molecular genetic studies, however, have revealed that this process is not the primary determinant of fruit softening, but may determine other aspects of fruit quality (Smith et al., 1988; Giovannoni et al., 1989; Schuch et al., 1991). Disassembly of the hemicellulose component of the wall during ripening is common to most fruit, although the extent varies between species and most likely reflects the degradation of a mixture of polysaccharides by multiple enzymes.

Expansins were originally considered to play a role on elongation growth, and high levels of expansin mRNAs have been detected in various growing tissues, including hypocotyls, roots, leaves, and young fruits (Shcherban et al., 1995; Cho and Kende, 1997; Reinhardt et al., 1998; Catala et al., 2000; Chen et al., 2001). However, in addition to growing tissues, the expression of expansin genes is also up-regulated in ripening fruit, including tomato, peach, strawberry, and pear (Rose et al., 1997; Civello et al., 1999; Hayama et al., 2000; Harrison et al., 2001; Hiwasa et al., 2003), where cell expansion does not occur, suggesting a role for expansins in the cell wall disassembly associated with fruit softening. It has been proposed that ripening-associated expansins might contribute to cell-wall degradation by increasing the accessibility of other cell-wall modifying proteins, such as the hydrolases PG or EGase, to structurally important cell-wall polymers (Rose and Bennett, 1999).

Grape berries are known to exhibit a double-sigmoidal growth curve, and rapidly soften at the beginning of the second growth cycle. The onset of this rapid softening is called "véraison" by viticulturists. Several researchers have reported changes in cell-wall components of grape berries during development and maturation. Yakushiji et al. (2001) showed that the neutral and acidic sugar contents of the pectin fraction decreased only after véraison, while the neutral sugar content of the hemicellulose decreased at véraison. Furthermore, hemicellulosic xyloglucan was markedly depolymerized at véraison.

In a previous study, we used cDNAs isolated from Kyoho grape berries to identify genes involved in cell wall modification using a subtraction method (Ishimaru and Kobayashi, 2002). In this study, we isolated the xyloglucan *endo*-transglycosylase (*VXET 1*) gene and demonstrated that the expression patterns of the *VXET 1* gene are closely related to berry softening at véraison. The work presented here continues our investigation of cDNAs likely to be involved in cell wall modification and fruit softening identified from the subtraction method (Ishimaru and Kobayashi, 2002). Here, we present the identification and characterization of three expansin cDNAs. The sequence analysis, DNA gel blot analysis and detailed expression profiles throughout fruit development and in different tissues are presented.

Materials and methods

Plant material

Grape (*Vitis labruscana* cv. Kyoho) berries (flesh and skin) from plants grown in a greenhouse, were harvested at 2, 4 and 6 weeks after flowering (WAF). Berries were collected at the stage just before véraison, at véraison (about 7 WAF), when beginning to color (about 7 WAF), when half-colored (8 WAF), when fully reddish purple (9 WAF), and when fully black/ripe (12 WAF). In grapes, the onset of berry softening and color formation differ among individual berries within a bunch and therefore includes berries at various stages of development. For accurate analysis of expansin gene expression at individual developmental stages, we collected berries at separate times from the same bunches. The berries were immediately frozen in liquid nitrogen and stored at −80° until use.

Roots, tendrils, young leaves (expanded to about 6 cm), flowers, and seeds were also collected from Kyoho vines.

Construction of a cDNA library and screening

To construct a cDNA library, we isolated total RNA from mature berries (12 WAF, flesh and skin), according to Loulakakis et al. (1996). mRNA was prepared using oligo (dT)-Latex (Oligotex-dT30 super, Takara). Double-stranded (ds) cDNA was synthesized from 5 μg of mRNA and ds cDNAs were cloned into a Uni-ZAP XR vector using a ZAP-cDNA synthesis kit (Stratagene). To screen for expansin clones, a partial-length cDNA of expansin labeled with horseradish peroxidase using an ECL direct nucleic acid labeling system (Amersham) was used as a probe. The partial-length cDNA clone was isolated from a subtractive library described previously (Ishimaru and Kobayashi, 2002). The library was made by subtraction between cDNAs from berries just before véraison and those at véraison using a PCR-select cDNA subtraction kit (Clonetech, USA). After subtraction, the remaining cDNAs expected to be véraison-specific were cloned into a pCR II vector (Invitrogen). Two cDNA clones for expansin were obtained by random sequencing, and one of them was used as a probe to screen the cDNA library. Approximately 100,000 recombinant phages of the library, packaged with a ZAP-cDNA Gigapack III gold cloning kit (Amersham), were screened. After a second round of screening, positive clones were converted to pBluescript phagemids and sequenced.

RNA and DNA gel blot analysis

For RNA gel blot analysis, 20 μg total RNA isolated from the stored samples was separated by electrophoresis on 1.2% agarose gels containing 0.66 M formaldehyde and transferred to Toropilon-Plus nylon membranes (Tropix). The membranes were hybridized with probes labeled with digoxigenin (DIG) using a PCR-DIG probe synthesis kit (Roche Diagnostics). The 3′-untranslated regions from the three expansin full-length cDNAs were used as gene-specific probes. Blots were hybridized for 16 h at 50° in a DIG Easy Hyb hybridization buffer (Roche Diagnostics), washed twice in 2 SSC (150 mM NaCl and 15 mM tri-sodium citrate, pH 7.0), 0.1% SDS at room temperature for 5 min each, and twice in 0.1 SSC, 0.1% SDS at 68° for 15 min. Target RNA was detected according to the manufacturer's protocol (Roche Diagnostics).

For DNA gel blot analysis, 2.5 μg gDNA, isolated from leaves according to the procedure of Kobayashi et al. (1996), was digested with appropriate restriction endonucleases, separated by electrophoresis on a 1% agarose gel in TAE (40 mM Tris–acetate, pH 8.0, 1 mM EDTA) buffer and transferred to nylon membranes. The membranes were hybridized using the same probes used for RNA gel blot analyses for 16 h at 42° in a DIG Easy Hyb buffer. Membrane washing and detection of target DNA was performed under the same conditions used for RNA gel blot analysis.

Results

Identification and characterization of expansin cDNAs

Cell-wall modification-related cDNA clones were isolated from a subtractive library made by subtraction between cDNAs from berries just before véraison and those at véraison (Ishimaru

```
                        Signal Peptide
                                                                    +
Vlexp1    1:MTLVG-------LFLVGFLSMVSSVHGQ--GWINAHATFYGGGDASGTMGGACGYGNLYS  51
Vlexp2    1:MATAAFSSISLALFLFC-LCLQGT-NGDYGGWEGGHATFYGGGDASGTMGGACGYGNLYS  58
Vlexp3    1:MATAAFSSISLALFLFC-LCLQGT-NGDYGGWEGGHATFYGGGDASGTMGGACGYGNLYS  58
                                 +  +    +      +          +
Vlexp1   52:EGYGTNTAALSTALFNNGLSCGSCYEIKCVNDGKWCLPGSIVITATNFCPPNNALPNNAG 111
Vlexp2   59:QGYGTNTAALSTALFNSGLSCGACYEMKCNDDPKWCLPGTLTVTATNFCPPNLALSNTNG 118
Vlexp3   59:QGYGTNTAALSTALFNSGLSCGACYEMKCNDDPKWCLPGTLTVTATNFCPPNLALSNTNG 118
              +                                 +
Vlexp1  112:GWCNPPLHHFDLSQPVFQHIAQYRAGIVPVSYRRVPCRRRGGIRFTINGHSYFNLVLITN 171
Vlexp2  119:GWCNPPLQHFDLAEPAFLQIAQYRAGIVPVSFRRVPCVKKGGIRFTINGHSYFNLVLITN 178
Vlexp3  119:GWCNPPLQHFDLAEPAFLQIAQYPSWNRTCVFQRVPCVKKGGIRFTINGHSYFNLVLITN 178
                                *     *   *
Vlexp1  172:VGGAGDVHAVAIKGSRTGWQSMSRNWGQNWQSNTYLNGQSLSFKVTTSNGHTIVSYNCVP 231
Vlexp2  179:VAGAGDVRAVSIKGSKTGWQPMSRNWGQNWQSNSYLNGQTLSFQVTASDGRTMTSLNVAP 238
Vlexp3  179:VAGAGDVRAVSIKGSKTGWQPMSRNWGQNWQSNSYLNGQTLSFQVTASDGRTMTSLNVAP 238
              *
Vlexp1  232:AHWSFGQTFSGAQFR                                              246
Vlexp2  239:AGWQFGQTYEGAQF-                                              252
Vlexp3  239:AGWQFGQTYEGAQF-                                              252
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Figure 1. Alignment of the deduced amino acid sequences of *Vlexp1*, *2* and *3*. Dashes indicate gaps introduced for maximal alignment. Conserved cysteine residues and tryptophan residues are indicated by crosses (+) and asterisks (*), respectively. A black box indicates an HFD motif region.

and Kobayashi, 2002). Two expansin homologues (partial-length cDNAs) were isolated from the subtractive library. Preliminary analysis of the expression of one of the homologues was assessed by RNA gel blot analysis during berry development. Expression of the homolog was slightly detected before véraison and markedly increased at véraison, indicating that it was a promising candidate for further study (data not shown).

To isolate full-length expansin cDNAs, a cDNA library constructed from mature berries of Kyoho was screened using the partial expansin cDNA from the subtractive library. Three full-length cDNA clones encoding three unique expansin open reading frames (Vexp1, 2 and 3) were isolated from the cDNA library (Fig. 1). The open reading frames ranged from 246 to 252 amino acids, with predicted molecular masses of 26.42–26.86 kDa and a pI of 7.46–9.15 (Table 1). Using SignalP, the three expansin open reading frames were predicted to have signal sequences and the cleavage sites were predicted to be at amino acid 20 or 25 (Table 1). In addition, the three expansins described here included several characteristics shared with previously identified expansins (Shcherban et al., 1995; Link and Cosgrove, 1998): including eight typical cysteine residues in the N-termini region of the proteins, the HFD motif in the central region and four tryptophan residues near the C-termini region (Fig. 1).

Phylogenetic analysis

A phylogenetic tree was generated from the deduced amino acid sequence alignment of the three expansins and 26 other expansin homologs (Fig. 2). Four distinct groups were identified (A, B, C, and D), confirming a previous report by Link and Cosgrove (1998). Vlexp2 and Vlexp3 aligned within group B, which contains several expansin genes that are expressed in ripe fruit, such as apricot (PaExp1 and PaExp2), peach (PpExp1), sweet cherry (PruavExp1), and strawberry (FaExp2). Vlexp1 aligned to group C, which contains LeExp5 (expressed in immature fruit, flowers, and shoots of

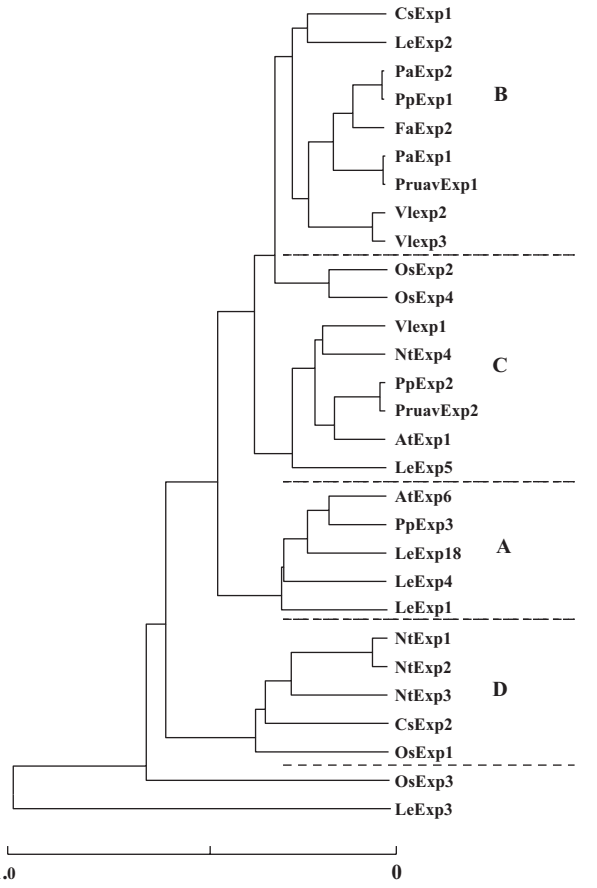

Figure 2. Phylogenetic comparison of expansin genes using the deduced amino acid sequences of the complete coding region. Alignments created by the UPGMA clustering method based on amino acid sequence; CsExp1 (U30482), CsExp2 (U30460) from *Cucumis sativus*, LeExp1 (U82123), LeExp2 (AJ239068), LeExp3 (AF059487), LeExp4 (AF059488), LeExp5 (AF059489), LeExp18 (AJ004997) from *Lycopersicon esculentum*, PaExp1 (U93167), PaExp2 (AF038815) from *Prunus armeniaca*, PpExp1 (AB029083), PpExp2 (AB047518), PpExp3 (AB047519) from *Prunus persica*, FaExp2 (AF159563) from *Fragaria ananassa*, PruavExp1 (AF297521), PruavExp2 (AF297522) from *Prunus avium*, OsExp1 (Y07782), OsExp2 (U30477), OsExp3 (U30479), OsExp4 (U85246) from *Oriza sativa*, AtExp1 (U30476), AtExp6 (U30480) from *Arabidopsis thaliana*, NtExp1 (AF049350), NtExp2 (AF049351), NtExp3 (AF049352), NtExp4 (AF049353) from *Nictiana tabacum*.

Table 1. Nucleotide and deduced amino acid sequences data on three expansin cDNAs identified in grape berry

	Full length (bp)	ORF (amino acid)	M.W. (kDa)	PI	DDBJ accession number
Vlexp-1	1245	246	26.42	9.15	AB104442
Vlexp-2	1280	252	26.72	8.02	AB104443
Vlexp-3	1175	252	26.86	7.46	AB104445

The theoretical molecular mass and pI were calculated using the ExPASy program.

tomato) and PruavExp2 (expressed in ripening sweet cherry fruit). No grape expansins aligned to group A, which contains LeExp1 (shown to be related to softening during ripening of tomato fruit), and LeExp4 (expressed in immature fruit and flowers of tomato).

DNA gel blot analysis

The gene-specific 3′untranslation regions (3′ UTR) of Vlexp1, 2 and 3 were used as probes (Vlexp1; 445 bp, Vlexp2; 380 bp, Vlexp3; 365 bp, there are no restriction site in these probes, which was used to restrict enzyme) for DNA gel blot analyses to determine the copy number of these genes in the grape genome. Vlexp1 hybridized to multiple fragments, suggesting that there is more than one copy or that there is cross hybridization to other expansin genes with nearly identical 3′ UTR sequences. Vlexp2 and Vlexp3 showed similar hybridization patterns, however the intensity of the bands differed. For example, the HindIII digest of Vlexp2 has 3 very faint bands and 2 sharp bands, while the same digest for *Vlexp3* resulted in 5 clearly detectable bands. Due to the results with *Vlexp2 and 3*, cross-hybridization was assessed among the cDNA probes and *Vlexp2 and 3* were found to slightly cross-hybridize to each other (data not shown). The results suggest that *Vlexp2 and 3* occur as single copy genes, but cross hybridize to each other slightly under the conditions tested.

Expression profile of *Vlexp1, 2 and 3*

To examine the potential role of expansins in berry development and maturation, *Vlexp* mRNA levels were evaluated in berries at nine developmental stages. Each *Vlexp* mRNA showed a unique accumulation pattern during berry development (Fig. 4). *Vlexp1* mRNA was detected throughout all the stages tested and accumulated to the greatest degree in berries at véraison and at the half-colored stage. *Vlexp3* mRNA accumulation was nearly identical to *Vlexp1*, except that no *Vlexp3* mRNA was detected at 2 WAF. Similarly, the *Vlexp2* mRNA was detected throughout berry development and maturation; however, its level started to increase at 4 WAF and continued to increase through véraison. Interestingly, *Vlexp2* mRNA accumulated to a very high level before véraison and increased even more as the berries matured. In contrast to *Vexp2*, *Vlexp1* and *Vlexp3* mRNA levels decreased during the later stages of maturation.

The expression patterns of the three expansin genes in other tissues of Kyoho vines were also examined (Fig. 5). *Vlexp1* mRNA was detected in flowers, tendrils, and leaves, and *Vlexp2* mRNA accumulated in all tissues tested. *Vlexp3* transcript was not detected in the tissues examined and therefore is likely to have fruit-specific expression.

Discussion

In grape berry softening, the rapid softening that takes place during véraison has been carefully studied in terms of changes in cell-wall components and at the gene expression level. A previous study suggested that the *VXET1* gene plays a critical role in berry softening at véraison in the Kyoho grape (Ishimaru and Kobayashi, 2002). The deduced amino acid sequence of *VXET1* showed 73.5% identity with the corresponding XET (*NXG 1*) from nasturtium (*Tropaeolum majus*), which has been shown to have *endo*-glucanase activity. We suggested that the *VXET1* product cleaves the cellulose–xyloglucan network of the cell wall and may play a role in softening of Kyoho grape berries at véraison.

In this study, we isolated three expansin cDNA clones (*Vlexp1*, *2*, and *3*) from mature grape berries. It is well known that expansins are encoded by a large multigene family in all plants reported to date. Based on phylogenetic analysis and shared intron patterns, expansins have been classified into three subfamilies, α, β, and γ-expansins (Li et al., 2002). *Vlexp1*, *2* and *3* belong to the α-expansin subfamily. In *Arabidopsis*, 38 expansin genes have been reported (Whitney et al., 2000), and 12 expansin genes have been identified in tomato. In addition, six expansin clones were isolated from strawberry (Civello et al., 1999; Harrison et al., 2001), seven from pear (Hiwasa et al., 2003), and three from peach (Hayama et al., 2000) and their expression patterns were characterized. DNA gel blot analysis of the grape expansin genes revealed unique patterns for each of the three specific probes (Fig. 3). The pattern of *Vlexp2* and *Vlexp3* were similar. However, differences in the expression patterns of *Vlexp2* and *Vlexp3* confirm that they are different genes (Fig. 5). Observations from work carried out in our laboratory suggest that there are at least ten expansin genes in the grape vine (data not shown).

RNA gel blot analysis was performed to characterize the expression patterns of the three expansin genes in berries at various developmental stages and in several tissues. There were essentially two expression patterns in berries for *Vlexp* genes: 1) expression increased with berry

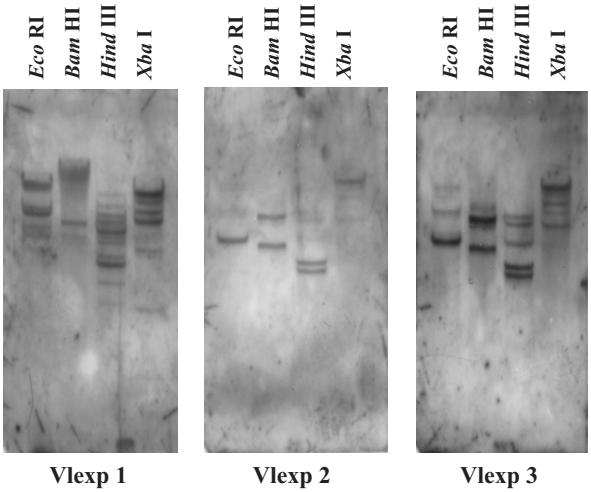

Figure 3. DNA gel blot analysis. Genomic DNA (5 μg per lane) was digested with the indicated restriction enzymes. Gel blots were hybridized with DIG-labeled probes prepared from the 3′-untranslation region of each expansin cDNA.

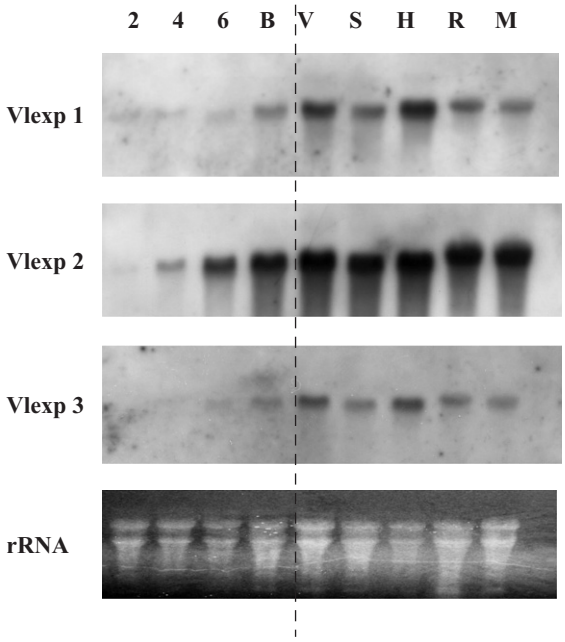

Figure 4. RNA gel blot analysis. Changes in the accumulation of three expansin genes during grape berry development and maturation. Total RNA (10 μg per lane) was separated by electrophoresis and transferred to nylon membranes. The membrane was hybridized with a DIG-labeled probe made using the 3′-untranslated region for each expansin cDNA. Capital letters indicate the stages (2, 4 and 6; 2, 4 and 6 weeks after flowering, B; just before véraison, V; at véraison (about 7 WAF), S; beginning to color (about 7 WAF), H; half-colored (8 WAF), R; fully reddish purple (9 WAF), M; fully black/ripe (12 WAF). A vertical dotted line means that the fruit softening just started. Ethidium bromide stained gel prior to blotting is shown below the blot as an approximate loading control.

development up to the half-colored stage and then decreased until the mature stage (*Vlexp1* and *Vlexp3*) and 2) expression increased strongly before véraison, peaked at véraison and remained at high levels to the maturation stage (*Vlexp2*).

Expansins are cell-wall-localized proteins that induce loosening of isolated plant cell walls *in vitro* in a pH-dependent manner, but exhibit no detectable hydrolase or transglycosylase activity (McQueen-Mason et al., 1993; McQueen-Mason and Cosgrove, 1994, 1995). Based on biochemical and biophysical evidence, expansins have been suggested to bind at the interface between cellulose microfibrils and matrix polysaccharides in the cell wall, disrupting hydrogen bonds within this polymeric network (McQueen-Mason and Cosgrove, 1994, 1995). Additional *in vitro* studies suggested that expansins act on the cellulose-xyloglucan framework (Brummell et al., 1999), although it has not been established that this represents the site of action *in vivo*. Furthermore, Brummell et al. (1999) showed that altering expansin expression in transgenic tomato fruit affected fruit firmness. Expansins have been proposed to regulate the accessibility of cell-wall-degradation enzymes to their polymer substrates in a model in which synergistic enzyme action is probably crucial for coordinated cell-wall disassembly in both growing vegetative tissues and ripening fruit (Rose and Bennett 1999). Hiwasa et al. (2003) observed that two pear PG genes were expressed in a similar pattern to that of four ripening-related expansin genes during pear fruit ripening and suggested that PG might act cooperatively with expansin to diminish the firmness of the fruit. In peach fruit, Hayama et al. (2000) also suggested that the transcript of PG mRNA was induced simultaneously with *PpExp3* in "Manami" fruit, which showed a reduction in firmness.

In Kyoho grape berries, Yakushiji et al. (2001) examined the change in cell-wall components and their molecular weights during berry softening. They reported that neutral and acidic sugar levels of the pectin fraction decreased only after the véraison stage, while the neutral sugar content of the hemicellulose fraction decreased at véraison. The cellulose content constantly decreased during berry softening, but a large decrease was observed at véraison. They also showed that the molecular masses of pectic and hemicellulosic polysaccharides began to decrease before véraison and up to véraison. In particular, hemicellulosic xyloglucan was markedly depolymerized from before véraison to véraison. Among all of the genes,

coding for cell-wall-modifying enzymes, we identified from our subtractive libraries (Ishimaru and Kobayashi, 2002), we observed that only the xyloglucan endo-transglycosylase (XET) gene is expressed in a similar manner to *Vlexp2* (Fig. 4). Therefore, the cooperative expression of *Vlexp2* and possibly *Vlexp1* and *3* with the XET gene may play an important role in grape berry softening before and at véraison (Fig. 5).

Vlexp1, which belongs to the phylogenetic tree subgroup C, was detected at an early growing stage when berries enlarge by cell division. In addition to *Vlexp1*, the expression of *Vlexp2*, which belongs to subgroup B, was also observed at an early growing stage. Interestingly, *Vlexp1* and *Vlexp2* were expressed at high levels in leaves, tendrils, roots, flowers, and seeds, although no expression of *Vlexp1* was detected in roots and seeds. This result suggests that these genes may be correlated with cell division and/or cell expansion. The possibility that these expansin genes play a role in fruit softening cannot be eliminated, when the potentially synergistic action of expansins is taken into consideration. However, it is more likely that these genes have a general role in cell wall modification during fruit development and maturation.

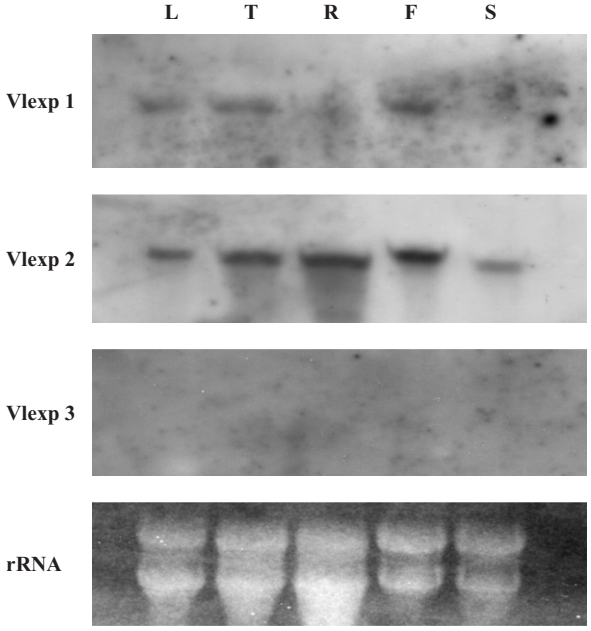

Figure 5. RNA gel blot analysis of various tissues of Kyoho vines. Total RNA from leaves (L), tendrils (T), roots (R), flowers (F), and seeds (S) were used. The membrane was hybridized with a DIG-labeled probe made using the 3′-untranslated region for each expansin cDNA. Ethidium bromide stained gel prior to blotting is shown below the blot as an approximate loading control.

The findings obtained in this study show that at least three expansin genes are associated with berry development and maturation. The *Vlexp2* product may be closely related to berry softening at véraison; it is plausible that *VXET 1* and *Vlexp3* products work synergistically and cleave the cellulose–xyloglucan network of the cell wall and contribute to softening of *Kyoho* grape berries at véraison. Further, analysis using transgenic plants with altered *Vlexp* expression may help to elucidate the role(s) of expansin in grape berry softening.

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